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Roles of *Mycobacterium smegmatis* D-Alanine:D-Alanine Ligase and D-Alanine Racemase in the Mechanisms of Action of and Resistance to the Peptidoglycan Inhibitor D-Cycloserine†

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D-Cycloserine (DCS) targets the peptidoglycan biosynthetic enzymes D-alanine racemase (Alr) and D-alanine:D-alanine ligase (Ddl). Previously, we demonstrated that the overproduction of Alr in *Mycobacterium smegmatis* determines a DCS resistance phenotype. In this study, we investigated the roles of both Alr and Ddl in the mechanisms of action of and resistance to DCS in *M. smegmatis*. We found that the overexpression of either the *M. smegmatis* or the *Mycobacterium tuberculosis ddl* gene in *M. smegmatis* confers resistance to DCS, but at lower levels than the overexpression of the *alr* gene. Furthermore, a strain overexpressing both the *alr* and *ddl* genes displayed an eightfold-higher level of resistance. To test the hypothesis that inhibition of Alr by DCS decreases the intracellular pool of D-alanine, we determined the alanine pools in *M. smegmatis* wild-type and recombinant strains with or without DCS treatment. Alr-overproducing strain GPM14 cells not exposed to DCS displayed almost equimolar amounts of L- and D-alanine in the steady state. The wild-type strain and Ddl-overproducing strains contained a twofold excess of L- over D-alanine. In all strains, DCS treatment led to a significant accumulation of L-alanine and a concomitant decease of D-alanine, with approximately a 20-fold excess of L-alanine in the Ddl-overproducing strains. These data suggest that Ddl is not significantly inhibited by DCS at concentrations that inhibit Alr. This study is of significance for the identification of the lethal target(s) of DCS and the development of novel drugs targeting the D-alanine branch of mycobacterial peptidoglycan biosynthesis.

The bacterial cell wall is an ideal target for drug design since similar structures and biosynthetic pathways are absent from mammalian hosts. The lipid-rich mycobacterial cell wall acts as an efficient permeability barrier (4). Peptidoglycan, the backbone of this structure, contains the D-amino acids D-alanine, D-glutamate, and diaminopimelate, which may contribute to its stability against proteolytic degradation. D-Alanine is one of the central molecules of the cross-linking step of peptidoglycan assembly. Peptidoglycan biosynthesis in mycobacteria follows pathways similar to those in other eubacteria (2). There are three enzymes involved in the D-alanine branch of peptidoglycan biosynthesis: the pyridoxal phosphate-dependent D-alanine racemase (Alr), the ATP-dependent D-alanine:D-alanine ligase (Ddl), and the ATP-dependent D-alanine:D-alanine-adding enzyme (MurF) (32). D-Cycloserine (DCS; 4-amino-3-isoxazolidinone) is a rigid analog of D-alanine and targets both Alr and Ddl in *Escherichia coli* (16, 19). DCS also inhibits *Mycobacterium tuberculosis* Alr and Ddl enzymes (9, 28), suggesting that both Alr and Ddl are targets of DCS in mycobacteria.

DCS is effective against mycobacteria and is recommended to treat multidrug-resistant *M. tuberculosis* in the DOTS-Plus management plan (13, 35). However, undesirable side effects restrict its use in human chemotherapy (36). Nonetheless, the potent bactericidal effect of DCS against mycobacteria makes this drug an attractive prototype compound to develop novel antimycobacterial agents. In addition, identification of the lethal target(s) of DCS action would allow for the rational design of new antimycobacterial drugs, structurally related or unrelated to DCS, targeting enzymes of the D-alanine pathway of peptidoglycan biosynthesis. Moreover, this type of inhibitors may weaken the cell wall and act synergistically with other antimicrobial agents (21). In the early 1970s, David (8) isolated and characterized step-wise DCS-resistant *M. tuberculosis* mutants that showed either normal or reduced cellular permeability to DCS and speculated that Alr plays only a minor role in the mechanism of action of DCS. *Mycobacterium smegmatis*, a nonpathogenic species, is a useful model to study drug resistance mechanisms in pathogenic mycobacteria, especially when conserved cellular processes are involved (23, 31). Peteroy et al. (20) described the isolation and characterization of an *M. smegmatis* mutant resistant to both DCS and vancomycin though the molecular basis of the resistance mechanism remains unknown. In our studies, a spontaneous DCS-resistant *M. smegmatis* mutant with a promoter-up mutation in the *alr* gene, resulting in the overproduction of the Alr enzyme, was identified (5). Alr was shown to be inhibited by DCS in a concentration-dependent manner, and DCS resistance could be conferred to pathogenic mycobacteria carrying the *M. smegmatis alr* gene in a multicopy plasmid. In addition, DCS was shown to competitively inhibit the native Ddl enzyme from *M. tuberculosis* (9). Belanger et al. (3) reported the characterization of a temperature-sensitive *M. smegmatis* mutant with a single amino acid substitution in Ddl. The mutant was more susceptible to DCS, and the temperature sensitivity phenotype was due to the decreased activity of the mutated enzyme.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference and/or source
E. coli XL10-GOLD	Tet^r Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r) Amy Cam^r]	Stratagene
M. smegmatis mc^{2}155	High-transformation *M. smegmatis* mutant derived from mc^{2}6	25
M. smegmatis GPM2	mc^{2}155(pMV262)	5
M. smegmatis GPM14	DCS-resistant mutant derived from mc^{2}155; overproducing Alr	5
M. smegmatis GPM259	mc^{2}155(pBUN250); overproducing Ddl	This work
M. smegmatis GPM260	GPM14(pBUN250); overproducing both Alr and Ddl	This work
M. smegmatis GPM265	mc^{2}155(pBUN276); overproducing *M. tuberculosis* Ddl	This work
M. smegmatis GPM198A	mc^{2}155(pBUN128A)	This work
pCR2.1	TA cloning vector	Invitrogen
pMV262	*E. coli-Mycobacterium* shuttle plasmid	MedImmune, Inc.; 7
pBUN128A	pMV262 with a 1.4-kb insert containing the complete *M. tuberculosis ddl* gene inserted at the *Eco*RI site	This work
pBUN172	Recombinant plasmid isolated from an *M. smegmatis* genomic library which hybridized with an internal fragment of the *ddl* gene	This work
pBUN250	pMV262 with a 1.4-kb insert containing the complete *M. smegmatis ddl* gene inserted at the *Eco*RI site	This work
pBUN276	pMV262 with the *M. tuberculosis ddl* gene inserted at the *Bam*HI/*Hin*dIII site; the *M. tuberculosis ddl* gene is fused with the DNA sequence corresponding to the first 6 codons of the *M. bovis* BCG *hsp60* gene	This work

Recently, we observed that *M. smegmatis alr* null mutants are not dependent on D-alanine for growth, suggesting the existence of another pathway for D-alanine biosynthesis (6). In addition, the *alr* null mutant is hypersusceptible to DCS, suggesting that a lethal target other than Alr is responsible for the bactericidal effect of DCS.

In this study, we investigated the roles of both Alr and Ddl in the mechanisms of action of and resistance to DCS in *M. smegmatis*. We demonstrate that Ddl activity is inhibited by DCS in a concentration-dependent manner. Overexpression of the *ddl* gene confers resistance to DCS but not to β-chloro-D-alanine (βCDA), an inhibitor of D-alanine racemase. Furthermore, a strain overexpressing both the *alr* and *ddl* genes displayed an increased level of resistance to DCS. Analysis of the intracellular alanine pools in wild-type and recombinant *M. smegmatis* strains suggested that Ddl activity is not significantly affected by DCS at concentrations inhibiting Alr.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown at 37°C in Luria-Bertani broth or agar. *M. smegmatis* strains were grown at 37°C with shaking (200 rpm; Innova 4300 incubator shaker; New Brunswick Scientific, Edism, N.J.) in Middlebrook 7H9 broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with albumin-dextrose complex (ADC) and 0.05% Tween 80 (M-ADC-TW broth). For analysis of the intracellular amino acid pools, *M. smegmatis* was grown in a minimal medium based on the formulation of Zygmunt (37) and modified as previously described (6). Tryptic soy agar base (Difco Laboratories, Detroit, Mich.) was used for growth of *M. smegmatis* on solid media. Transformations of *E. coli* and *M. smegmatis* were performed by electroporation with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) as described previously (14). The following antibiotics were added to the media when necessary: kanamycin (Sigma Chemical Co., St. Louis, Mo.) at 50 μg ml^{-1} or carbenicillin (Sigma) at 50 μg ml^{-1} for *E. coli* strains and kanamycin at 10 μg ml^{-1} for *M. smegmatis* strains.

Oligonucleotide primers, nucleic acid manipulations, and primer extension analysis. The oligonucleotide primers (Integrated DNA Technologies, Inc., Coralville, Iowa) for PCR amplification of the complete *ddl* gene from *M. smegmatis* were SMDDLCF (5′-CGC ATA AGG CCA GGT CAG-3′) and SMDDLCR (5′-CGA AAA ACC CGT CGT GC-3′). The primers for PCR amplification of the *ddl* gene from *M. tuberculosis* were DDLATBU (5′-GCT AAG TGC CGA TCG CAA G-3′) and DDLATBD (5′-ATA ACG CTG CTG CTG GGT C-3′) and TBDDLEXF (5′-CGG GAT CCG TGA GTG CTA ACG AC-3′) and TBDDLEXR (5′-CGG AAG CTT GTG CCG ATC GCA AGC-3′). The primers SMDDLPE (5′-AAA CGC TCC GGA TCG AGG TTG-3′) and TBDDLPE (5′-GAG ATG GCG TGC TCG TTG-3′) were used in primer extension analysis for the *ddl* mRNA of *M. smegmatis* and *M. tuberculosis*, respectively. PCR amplifications were performed in a Perkin-Elmer GeneAmp 9600 thermal cycler (Roche Molecular Systems, Branchburg, N.J.) by using the Expand high-fidelity PCR system (Roche Molecular Biochemicals, Indianapolis, Ind.). For restriction digestions, ligations, and agarose gel electrophoresis, standard procedures previously described (24) were followed. Total RNA from *M. smegmatis* strains was isolated by using RNAWIZ (Ambion, Inc., Austin, Tex.) with minor modifications (1). Primer extension analysis of the *ddl* mRNA was carried out as described previously (11). The oligonucleotide was radiolabeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Promega, Madison, Wis.), and the reactions were extended with Moloney murine leukemia virus reverse transcriptase (Promega). Radioactivity in primer extension products was quantified with a PhosphorImager by using ImageQuant, version 3.3 (Molecular Dynamics, Sunnyvale, Calif.).

***M. smegmatis* genomic library construction and cloning of the *M. smegmatis ddl* gene.** Chromosomal DNA from *M. smegmatis* mc^{2}155 was prepared as described previously (33). For library construction, chromosomal DNA was partially digested with *Sau*3AI, and fragments of 3.0 to 4.0 kb were purified from 0.8% agarose. This fraction was ligated with the *E. coli-Mycobacterium* shuttle plasmid pMV262 (7), linearized with *Bam*HI, and dephosphorylated. The ligation mixture was transformed into *E. coli* XL10-GOLD (Stratagene, La Jolla, Calif.), and approximately 6,000 recombinants were obtained for a theoretical representation of P as >99% of the *M. smegmatis* genome.

A recombinant clone carrying the *M. smegmatis ddl* gene was identified from a genomic library by colony hybridization using a species-specific probe. An internal fragment of the *M. smegmatis ddl* gene was amplified by PCR using a pair of degenerate primers, DDLF and DDLR, based on two signature peptides of bacterial Ddl enzymes (12). This amplified fragment was verified and radiolabeled with the Rediprime II labeling system (Amersham Pharmacia Biotech, Piscataway, N.J.). For screening the library, about 10,000 colonies from the library pool were plated, transferred to the NYTRAN nylon membrane (Midwest Scientific, Valley Park, Mo.), and screened with the labeled probe as described previously (24). After three rounds of screening, the recombinant plasmid pBUN172 was identified and confirmed to contain the full-length *ddl* gene. This sequence is identical to the sequence at GenBank with accession no. AF077728 (3) and the sequence from the unfinished *M. smegmatis* mc^{2}155 genome (http://www.tigr.org).

Drug susceptibility assays. MICs were determined by a microdilution method described previously (29) with minor modifications. Briefly, *M. smegmatis* cells were grown in M-ADC-TW to mid-exponential phase (optical density at 600 nm,

0.6 to 1.0). Approximately 10^5 CFU in 0.1 ml were inoculated in triplicate onto 96-well microplates containing serial twofold dilutions of inhibitory compounds. Plates were incubated at 37°C and examined daily. The MIC was defined as the minimal concentration of the drug or inhibitor that prevented visible bacterial growth after 48 h. Each MIC represents the consistent result from at least three independent cultures. Amikacin, DCS, βCDA, ethambutol, and vancomycin (all from Sigma) were prepared in sterile deionized water. Rifabutin (Amersham Pharmacia) was prepared in dimethyl sulfoxide (Fisher Scientific, St. Louis, Mo.). All further dilutions of each antibiotic were prepared in growth medium. Inhibition of colony formation by DCS was evaluated as described previously (5). Appropriate dilutions of exponentially growing *M. smegmatis* cells were plated onto agar containing 0 to 1,200 μg of DCS ml^{-1}. Colonies were counted after 5 days of incubation at 37°C. Statistical analysis was conducted using the SAS general linear model procedure (SAS Institute, Cary, N.C.).

Preparation of crude cell extracts from *M. smegmatis* strains. *M. smegmatis* cells were harvested at exponential phase and concentrated 50-fold in ice-cold 50 mM Tris-HCl (pH 8.0). Cells were disrupted with a French pressure cell (Thermo Spectronic US, Rochester, N.Y.) at 14,000 lb/in^2. The lysate was centrifuged at 4°C for 30 min at 30,000 × *g* to remove cell debris. The supernatant was subjected to ultracentrifugation at 4°C for 4 h at 110,000 × *g* to remove the membrane fraction. The recovered supernatant was dialyzed twice against 50 mM Tris-HCl (pH 8.0) at 4°C and sterilized by filtration through 0.22-μm-pore-size filters. The protein concentration was determined by using the *DC* protein assay (Bio-Rad) as recommended by the manufacturer.

Enzyme assays. The crude extract was assayed for Ddl activity by a modified thin-layer chromatography (TLC)-based method described previously (18). This procedure can quantitatively determine the amount of D-alanine:D-alanine dipeptide. Briefly, crude extracts (5 to 30 μg of total protein) were incubated at 37°C for 4 h in a 50-μl final volume containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM KCl, 50 mM unlabeled D-alanine, 6 mM ATP, 2.5 mM glutathione, and 5.0 μCi of [1-^{14}C]D-alanine (100 μCi/ml; ICN Biochemicals, Inc., Costa Mesa, Calif.). For inhibition assays, DCS was added to the final concentrations of 50, 100, 200, 400, and 800 μg ml^{-1}. Subsequently, 10 μl of the reaction mixture was applied to cellulose-backed TLC plates (Sigma), and ascending chromatography was developed in *n*-butanol–acetic acid–water (12:3:5) until the solvent reached the top of the TLC plate. The plate was dried at 100°C for 5 min, and the radioactivity corresponding to the position of the D-alanine and D-alanine:D-alanine dipeptide was measured with a PhosphorImager. Specific activity was calculated as micromoles of D-alanine consumed per milligram per minute. The alanine racemase activity was measured in the direction of the conversion of L-alanine to D-alanine by a coupled spectrophotometric method (34) with modifications (6). Specific activity was calculated as described previously (5).

Analysis of intracellular pools of amino acids. *M. smegmatis* cells were grown in minimal medium until exponential phase and split into two subcultures, and DCS was added to a final concentration of 75 μg ml^{-1} to one of the subcultures. After 2 h of incubation, cells were harvested at 4°C by centrifugation, washed twice with ice-cold double-distilled water, and concentrated 50-fold. Cells were sonicated in a salt-ice-water bath with a Vibra-Cell model VC600 sonicator (Sonic and Materials, Inc., Danbury, Conn.) for 10 min at 80% power output and 50% duty cycle. The lysate was centrifuged at 4°C for 30 min at 30,000 × *g* to remove bacterial debris. Protein was removed from the supernatant by serial passages through YM-10 and YM-3 Centricon concentrators (Millipore Corp., Bedford, Mass.). Determination of the abundance of individual amino acids was performed at the Amino Acid Geochronology Laboratory of Northern Arizona University (Flagstaff, Ariz.) by a reverse-phase high-performance liquid chromatography (HPLC) procedure described previously (15). This procedure is able to detect nine pairs of L- and D-amino acids in the subpicomole range. For each sample, the area of the peak representing L-glutamate constituted 50 to 60% of the total area. Thus, the abundance of each amino acid was calculated as the area under the corresponding peak and expressed relative to this pool.

RESULTS

Overexpression of the *M. smegmatis* and *M. tuberculosis ddl* genes in recombinant *M. smegmatis*. In previous studies, a genomic library from a DCS-resistant mutant constructed in a multicopy plasmid was introduced into the wild-type *M. smegmatis* strain and clones resistant to 300 μg of DCS ml^{-1} were selected and isolated (5). Using this strategy, we did not identify a recombinant clone carrying the *ddl* gene. Since target overproduction determines a drug resistance phenotype, this outcome was unexpected. To exclude any possible bias in this library, we also screened a *M. smegmatis* cosmid library in a similar manner, but we were still unable to identify a DCS-resistant clone carrying the *ddl* gene. Lowering the DCS concentration resulted in a high background of DCS-sensitive colonies. These data suggest that either the overexpression of the *ddl* gene is toxic to the host or that the level of overexpression is not sufficient to confer a selectable resistance phenotype under the selection conditions described. To test these hypotheses, the *M. smegmatis* and *M. tuberculosis ddl* genes were amplified from genomic DNA and cloned into the *E. coli-Mycobacterium* shuttle vector pMV262, carrying a kanamycin resistance marker. Recombinant plasmids were introduced into *M. smegmatis*, and kanamycin-resistant transformants were isolated.

The *M. smegmatis ddl* gene was isolated from a genomic library as described in Materials and Methods. The DNA fragment containing the *ddl* gene coding sequence, including 190 bp of the 5′ flanking region and 35 bp of the 3′ flanking region, was amplified by PCR and ligated into the *Eco*RI site of pMV262 to generate pBUN250. This plasmid was electroporated into *M. smegmatis* mc^2155, yielding GPM259. Since pMV262 is present in *M. smegmatis* at 5 to 10 copies (5), the *ddl* gene is expected to be overexpressed in the recombinant *M. smegmatis* strain due to a gene dosage effect. This was confirmed by primer extension analysis using total RNA isolated from mc^2155 and GPM259. Radiometric quantitation of the primer extension bands confirmed that GPM259 overproduced the *ddl* transcript by 25-fold compared to mc^2155. In both samples, two transcriptional start sites were detected, one immediately upstream and another 3 nucleotides upstream of the start codon (GTG) (Fig. 1A). Further analysis of the sequence immediately adjacent to the start codon did not reveal any significant RNA secondary structures, excluding the possibility that the two bands detected were the result of premature extension products. It has been found for the *Mycobacterium fortuitum blaF* (30) and 11 streptomycete genes (27) that transcription start sites are extremely close to the start codon. This may suggest that translation initiation in both mycobacteria and streptomycetes has less-stringent requirements for the recognition of a ribosome-binding site.

Following the same strategy, we constructed an *M. smegmatis* recombinant strain, GPM198A, carrying the plasmid pBUN128A, a pMV262-based plasmid containing the *M. tuberculosis ddl* gene coding sequence, including 140 bp of the 5′ flanking region and 130 bp of the 3′ flanking region. Total RNA isolated from GPM198A was subjected to primer extension analysis using oligonucleotides specific to either the *M. smegmatis* or the *M. tuberculosis ddl* gene. The results revealed that this strain overexpressed the endogenous *M. smegmatis ddl* gene rather than the plasmid-carried *M. tuberculosis ddl* gene (data not shown). To circumvent this problem, a different strategy was followed. Briefly, the *M. tuberculosis ddl* gene was amplified by PCR and cloned into pMV262, yielding pBUN276. In this construct, the *M. tuberculosis ddl* gene is fused in frame with the first six codons of the *Mycobacterium bovis* BCG *hsp60* gene and is under the control of its promoter. Total RNA isolated from the corresponding recombinant strain, GPM265, was subjected to primer extension analysis as described above. In this case, the chromosomally encoded *ddl*

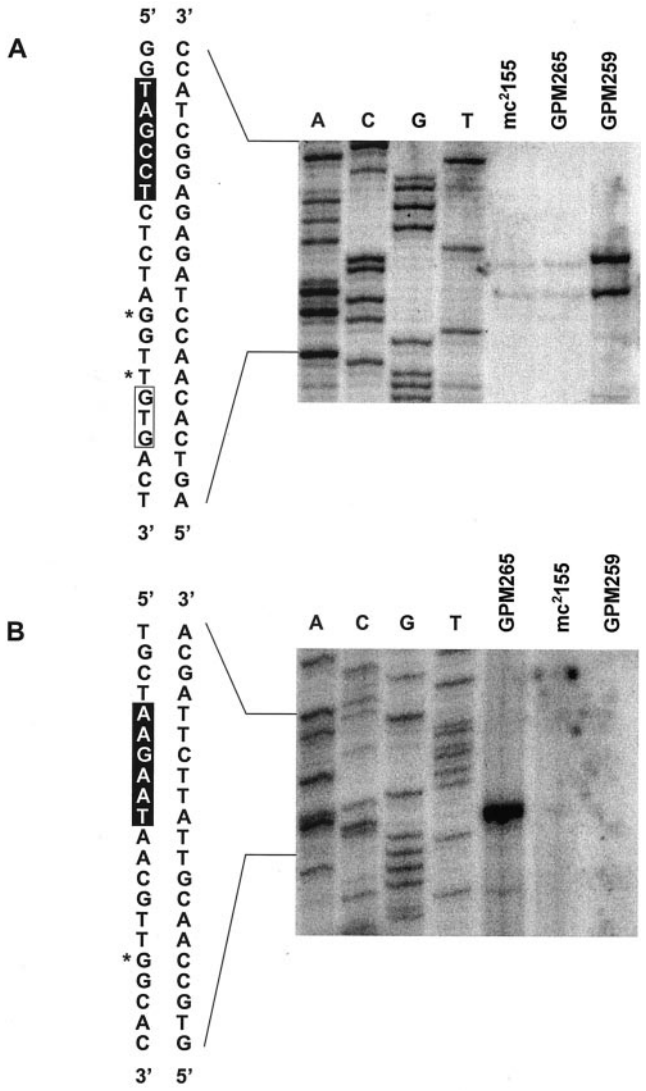


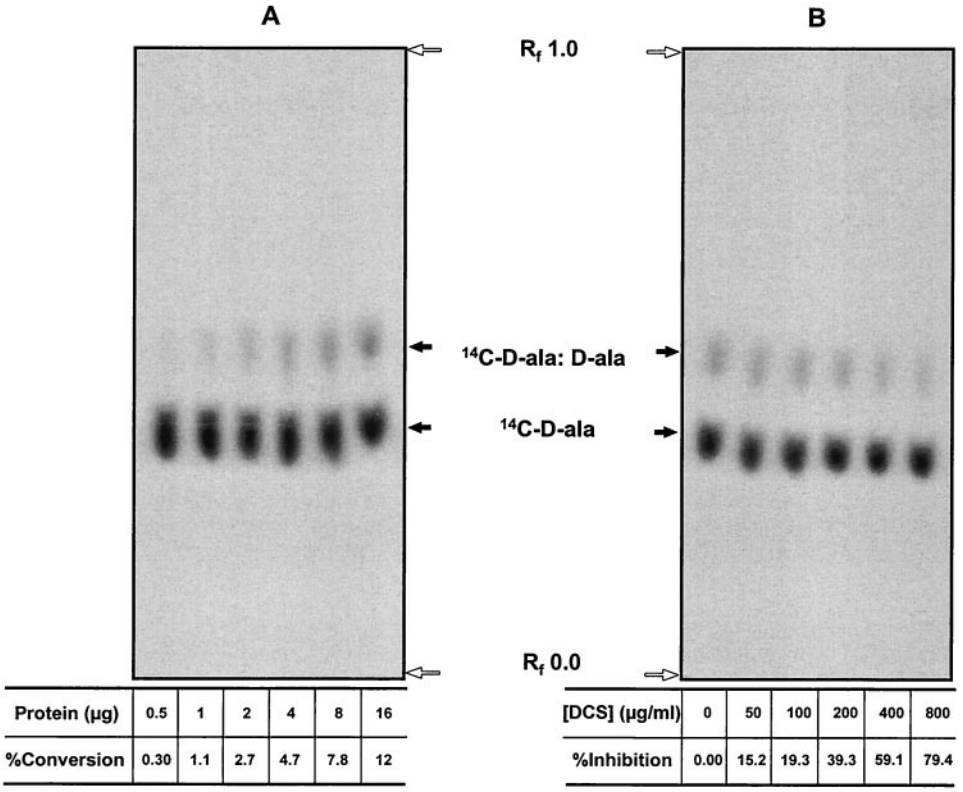
FIG. 1. Primer extension analysis of the *M. smegmatis* and *M. tuberculosis ddl* transcripts. Total RNA (40 μg) from mc^{2}155, GPM259, and GPM265 was annealed with primer SMDDLPE (A) or TBDDLPE (B) and extended as described in Materials and Methods. Lanes A, C, G, and T display a dideoxy sequencing ladder with the corresponding primer. Asterisks, transcriptional start sites. The putative −10 boxes are highlighted. The start codon (GTG) for the *M. smegmatis ddl* gene is boxed.

gene in GPM265 was expressed at a level similar to the level in wild-type mc^{2}155 (Fig. 1A), while the episomal *M. tuberculosis ddl* gene was expressed at an approximately 30-fold-higher level than the chromosomally encoded *ddl* gene (Fig. 1B). The transcriptional start site of the *M. tuberculosis ddl* gene located on pBUN276 was identified as the G nucleotide located 183 bp upstream of the start codon of the *hsp60* gene, consistent with previous observations (26). As expected, no *M. tuberculosis ddl* transcript was detected from mc^{2}155 or GPM259 total RNA.

Enzymatic characterization of *M. smegmatis* strains. The overexpression of the *M. smegmatis* or *M. tuberculosis ddl* gene was further confirmed by measuring Ddl enzyme activities. For this, Ddl-specific activities in cell crude extracts from mc^{2}155, GPM259, and GPM265 were determined by the TLC-based method described in Materials and Methods. A typical autoradiogram of the TLC-based Ddl enzyme assays for strain GPM259 is shown in Fig. 2A. Ddl-specific activities in crude extracts from GPM259 and GPM265 were about 35 and 30 times greater, respectively, than that in mc^{2}155 extracts (Fig. 3A). These increases in Ddl activity were consistent with the primer extension results. As a control, Alr enzyme activities were concurrently determined by a coupled spectrophotometric method as described in Materials and Methods. As expected, cell crude extracts from mc^{2}155, GPM259, and GPM265 displayed similar Alr activities (Fig. 3B), indicating that overexpression of the *ddl* gene is specific and does not alter the expression of the *alr* gene. Furthermore, there were no observed differences between the recombinant strains overproducing Ddl and the wild-type strain regarding growth rate, growth saturation density, and colony morphology, indicating that the overexpression of the *ddl* gene is not toxic to the host.

Previously, David et al. (9) reported that DCS competitively inhibits the *M. tuberculosis* Ddl enzyme. In this study, we performed in vitro inhibition assays to determine the Ddl-specific activities of crude extracts from mc^{2}155, GPM259, and GPM265 in the presence of increasing concentrations of DCS. A typical autoradiogram of the inhibition assay for the crude extract of GPM259 is shown in Fig. 2B. The degree of inhibition for the Ddl activities in these extracts increased proportionally to the DCS concentration (Fig. 4). No statistically significant differences between the extracts from mc^{2}155 and GPM259 were observed. However, though the inhibition of the Ddl activity in GPM265 crude extract followed a similar pattern, the percentage of inhibition was approximately 10% higher at each concentration of DCS ($P = 0.02$). Since the primer extension analysis indicated that more than 95% of the Ddl activity present in the crude extract of GPM265 is from the expression of the *M. tuberculosis ddl* gene, this slight difference may reflect a moderately higher sensitivity of the *M. tuberculosis* Ddl enzyme to DCS inhibition. In summary, these data indicate that both *M. smegmatis* and *M. tuberculosis* Ddl enzymes are inhibited by DCS in a concentration-dependent manner and provide further evidence that Ddl is a target of this drug.

Drug susceptibilities of *M. smegmatis* strains. To test whether Ddl overproduction confers a DCS resistance phenotype, we evaluated the susceptibilities of *M. smegmatis* strains overproducing Ddl to DCS. The MICs of DCS for mc^{2}155, GPM259, GPM265, and GPM14 were determined (Table 2). GPM14, overproducing Alr, is a spontaneous DCS-resistant mutant derived from mc^{2}155 (5). The MICs of DCS for strains GPM259 and GPM265 (both at 150 μg ml^{-1}) were twofold greater than that for mc^{2}155 (75 μg ml^{-1}) but were lower than that for GPM14 (300 μg ml^{-1}). Thus, recombinant strains overproducing Ddl showed increased levels of resistance to DCS but were not as resistant as the Alr-overproducing strain GPM14. The susceptibilities of these strains to βCDA, which interferes with the incorporation of D-alanine into the bacterial cell wall, were also determined (Table 2). βCDA, an analog of D-alanine, was reported to target bacterial alanine racemases and transaminases (17) and showed a synergistic effect with DCS against *M. tuberculosis* (10). In this study, only the strain overproducing Alr showed increased resistance to βCDA, as



Protein (μg)	0.5	1	2	4	8	16
%Conversion	0.30	1.1	2.7	4.7	7.8	12

[DCS] (μg/ml)	0	50	100	200	400	800
%Inhibition	0.00	15.2	19.3	39.3	59.1	79.4

FIG. 2. Autoradiograms of the TLC for typical Ddl assays. Ddl activities in crude extracts were determined as described in Materials and Methods. (A) Linear increase in the synthesis of the D-alanine:D-alanine dipeptide at increasing amounts of GPM259 cell extract. The percentage of conversion was defined as the percentage of the total radioactivity present in the [^{14}C]D-Ala:D-Ala dipeptide spot. (B) Percent inhibition of dipeptide formation (percent inhibition = 100 − 100 × [percent conversion$_{\text{with DCS}}$/percent conversion$_{\text{without DCS}}$]) in GPM259 cell extract (8 μg of total protein) at increasing concentrations of DCS.

the MIC for GPM14, 100 μg ml^{-1}, is fourfold higher than those for other strains with wild-type levels of Alr. These data are consistent with the mode of action of βCDA, which inhibits the Alr enzyme. In addition, susceptibilities to other antimicrobial agents targeting cell wall biosynthesis (ethambutol and vancomycin), RNA synthesis (rifabutin, a structural analog of rifampin with higher antimycobacterial activity), and protein synthesis (amikacin) of these *M. smegmatis* strains were identical (data not shown). The DCS resistance phenotype of the Ddl-overproducing strains was further confirmed by plating bacteria on solid agar containing various concentrations of DCS. Consistent with the MIC data, GPM259 and GPM265 are more resistant to DCS than the wild-type mc^{2}155 but less resistant than the Alr-overproducing strain GPM14 (Fig. 5). Furthermore, this type of intermediate level of resistance to DCS is not related to Alr, since crude extracts from both GPM259 and GPM265 contained a wild-type level of Alr activity (Fig. 3B). Therefore, overproduction of the Ddl enzymes leads to a DCS resistance phenotype in *M. smegmatis*. However, this level of resistance may not suffice for selection of DCS-resistant clones by following the strategy described above.

Construction and characterization of an Alr-Ddl-overproducing strain. It has been reported previously that a specific type of DCS-resistant *Streptococcus gordonii* mutants displayed elevated Alr and Ddl activities and that the level of resistance

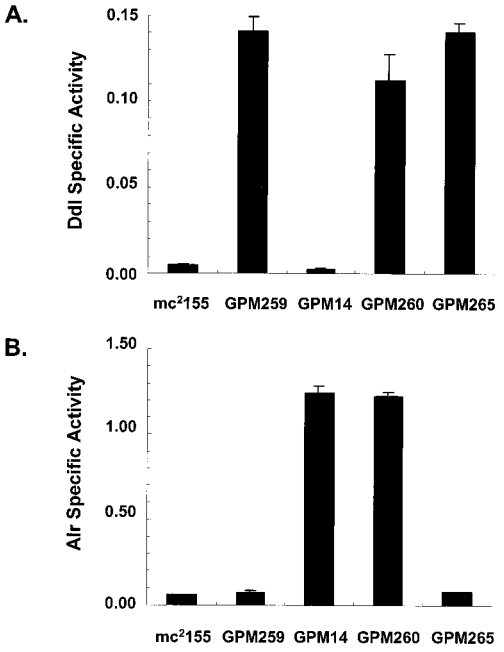


FIG. 3. Analysis of Dlr and Alr activities in *M. smegmatis* strains. Specific activities for Ddl (A) and Alr (B) in crude extracts were determined as described in Materials and Methods. Specific activities are expressed as micromoles of substrate (L-alanine for Alr enzyme assay and D-alanine for Ddl enzyme assay) per milligram per minute (means ± standard deviations of triplicate measurements).

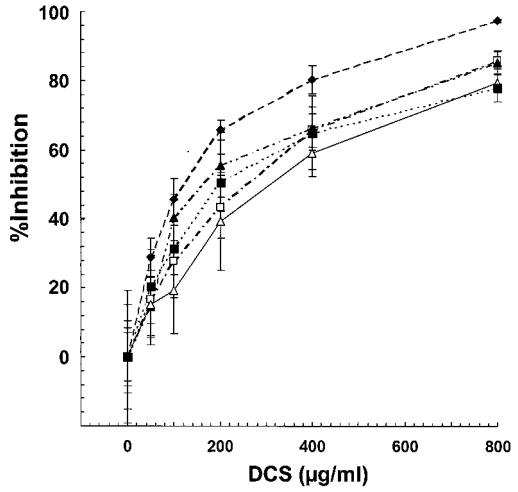


FIG. 4. Inhibitory effect of DCS on *M. smegmatis* Ddl activities. Enzyme activities were determined in cell crude extracts from mc^{2}155 (solid squares), GPM14 (solid triangles), GPM259 (open triangles), GPM260 (open squares), and GPM265 (solid diamonds) in the presence of increasing concentrations of DCS. Percent inhibition was calculated (percent inhibition = $100 - 100 \times [SA_{\text{with DCS}}/SA_{\text{without DCS}}]$; SA, specific activity) from three independent experiments. Statistical analysis was performed as described in Materials and Methods. The inhibition patterns can be divided into two groups (group I, mc^{2}155, GPM14, GPM259, and GPM260; group II, GPM265). A significant difference between groups was detected ($P = 0.02$), while no significant differences within group I were detected ($P = 0.28$).

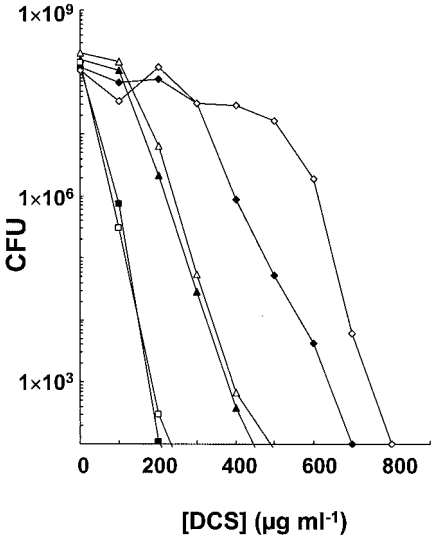


FIG. 5. Inhibition of colony formation by DCS for various *M. smegmatis* strains. The curves were generated from data of a representative experiment with mc^{2}155 (solid squares), GPM2 (open squares), GPM259 (open triangles), GPM265 (solid triangles), GPM14 (solid diamonds), and GPM260 (open diamonds). Statistical analysis for three independent experiments was performed as described in Materials and Methods. Susceptibilities to DCS can be divided into four groups (group I, mc^{2}155 and GPM2; group II, GPM259 and GPM265; group III, GPM14; group IV, GPM260). Significant differences between groups were detected (group I versus group II, $P = 0.003$; group II versus group III, $P = 0.025$; group III versus group IV, $P = 0.020$), while no significant differences within groups were detected ($P \geq 0.881$).

to DCS is higher than that for mutants with elevated Alr activity only (22). To test the effect of the overproduction of both Alr and Ddl in *M. smegmatis* on DCS susceptibility, we introduced the multicopy plasmid pBUN250 carrying the *ddl* gene into the Alr-overproducing strain GPM14. As expected, the resulting strain, GPM260, overproduced both Alr and Ddl as demonstrated by enzymatic characterization (Fig. 3). In addition, the degree of inhibition of the Ddl activities in extracts from GPM260 and its parental strain, GPM14, increased proportionally to the DCS concentration (Fig. 4). No statistically significant differences between these extracts were observed ($P = 0.28$). Note that the overproduction of Alr in GPM14 and GPM260 does not alter the degree of in vitro inhibition of the Ddl enzyme by DCS. Thus, increasing amounts of Alr protein do not seem to affect the inhibitory effect of DCS on Ddl activity. The susceptibilities of GPM260 to βCDA, ethambutol, vancomycin, rifabutin, and amikacin were identical to those of the parent strain, GPM14 (Table 2 and data not shown). However, GPM260, overproducing both Alr and Ddl, was significantly more resistant to DCS than the wild-type strain or strains overproducing only Alr or Ddl (Table 2 and Fig. 5).

TABLE 2. MICs of DCS *M. smegmatis* strains

Strain	Phenotype		MIC[a] (μg ml^{-1})	
	Alr	Ddl	βCDA[b]	DCS[c]
mc^{2}155	wt[e]	wt	25.0	75.0
GPM2	wt	wt	25.0	75.0
GPM259	wt	Overproduced	25.0	150
GPM14	Overproduced	wt	100	300
GPM260	Overproduced	Overproduced	100	600
GPM265	wt	Overproduced[d]	25.0	150

[a] MICs were determined in M-ADC-TW media by a microdilution method as described in Materials and Methods.

[b] A chi-square test was performed for MICs obtained from at least four independent cultures. Susceptibilities to βCDA can be divided into two groups (group I, mc^{2}155, GPM2, GPM259, and GPM265; group II, GPM14 and GPM260). Significant differences ($P < 0.001$) between these two groups were detected, while no significant differences ($P \geq 0.537$) within each group were detected.

[c] A chi-square test was performed for MICs obtained from at least seven independent cultures. Susceptibilities to DCS can be divided into four groups (group I, mc^{2}155 and GPM2; group II, GPM259 and GPM265; group III, GPM14; group IV, GPM260). Significant differences between these two groups were detected (group I versus group II, $P = 0.001$; group II versus group III, $P = 0.001$; group III versus group VI, $P = 0.008$), while no significant differences within each group were detected (group I, $P = 0.881$; group II, $P = 0.893$).

[d] More than 95% of the Ddl activity in GPM265 is due to the expression of the episomal copy of the *M. tuberculosis ddl* gene (see text).

[e] wt, wild type.

Analysis of the intracellular L- and D-alanine pools. The incorporation of D-alanine into peptidoglycan requires the sequential interconversion of L- into D-alanine followed by the formation of the D-alanine dipeptide, reactions catalyzed by Alr and Ddl, respectively. The interplay between D-alanine biosynthesis by Alr and its consumption by Ddl contributes to the determination of the intracellular level of this amino acid. Inhibition of Alr alone would decrease the intracellular pool of D-alanine. Therefore, in live bacilli exposed to sublethal concentrations of DCS (near the MIC for the wild-type strain), if Alr is inhibited to a greater extent than Ddl, a decrease in the

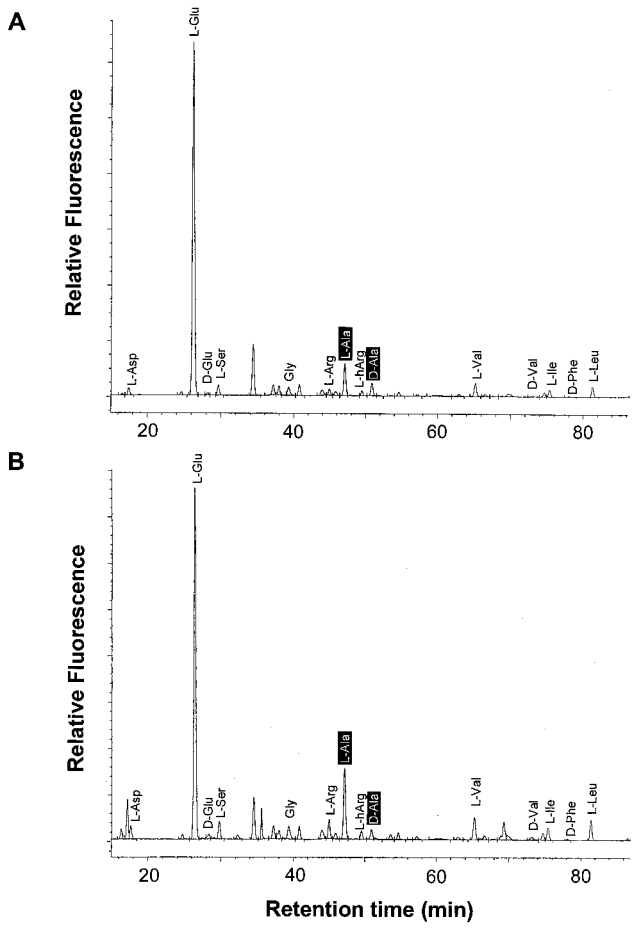
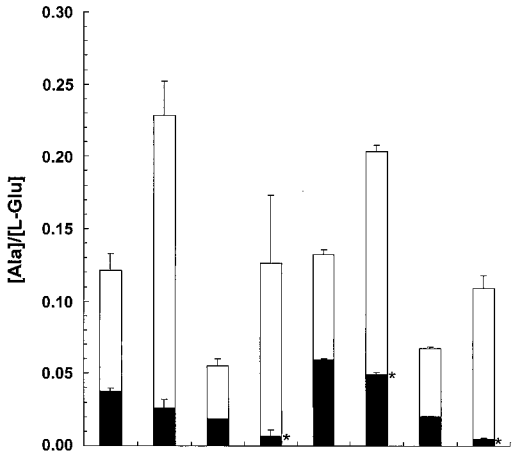


FIG. 6. Characteristic HPLC chromatographic profiles of *M. smegmatis* intracellular amino acid pools. Intracellular amino acid pools of *M. smegmatis* mc^{2}155 untreated (A) and treated with DCS (B) were prepared and analyzed by a reverse-phase HPLC method as described in Materials and Methods. The synthetic amino acid L-*homo*-arginine (L-hArg) served as an internal standard for quantifying the abundance of each individual amino acid. Peaks for various amino acids are indicated, with L- and D-alanine highlighted.



Strain	mc^{2}155		GPM259		GPM14		GPM260	
DCS	-	+	-	+	-	+	-	+
[L-ala]/[D-ala]	2.2	7.8	2.0	18	1.2	3.1	2.3	20

FIG. 7. Intracellular pool of alanine in *M. smegmatis* strains. Exponentially growing *M. smegmatis* cells in minimal media were split into two subcultures, and DCS was added to a final concentration of 75 μg ml^{-1} to one of the subcultures. Cells were harvested after 2 h of incubation, and intracellular amino acid pools were determined as described in Materials and Methods. The intracellular pool of alanine (full-length bars) is expressed relative to the L-glutamate pool ([Ala]/L-Glu]). The relative abundance of each stereoisomer in the pool is also shown by open (L-alanine) and solid (D-alanine) bars and indicated in the table at the bottom. Values shown are means ± standard deviations of three independent experiments. Analysis of variance was used to compare values for total alanine, L-alanine, and D-alanine pools in each strain for both DCS-treated and untreated cells. Significant differences were observed for the L-alanine pools ($P < 0.03$). For D-alanine pools, significant differences (asterisks) were observed only for strains GPM259 ($P = 0.05$), GPM14 ($P < 0.01$), and GPM260 ($P < 0.01$).

D-alanine pool would be expected. To test this hypothesis, we determined the intracellular alanine pools of mc^{2}155, GPM14, GPM259, and GPM260 with and without DCS treatment by a reverse-phase HPLC. This methodology allowed detection of D- and L-alanine, L-arginine, L-asparagine/aspartate, D- and L-glutamine/glutamate (Glx), glycine, L-isoleucine, L-leucine, D-phenylalanine, L-serine, and D- and L-valine from samples prepared from cell extracts as described in Materials and Methods (Fig. 6). It was observed that the L-Glx pool was the most abundant, probably reflecting its crucial role in nitrogen metabolism. Moreover, this pool displayed no significant variations upon DCS treatment ($P = 0.63$), so it was used as a standard to normalize all other intracellular pools.

Comparison of the amino acid pools in each strain upon DCS exposure revealed that this treatment had a specific effect on the intracellular alanine pools (Fig. 7). In all strains tested, DCS treatment led to a significant accumulation of L-alanine ($P < 0.03$) and a concomitant decrease of D-alanine. The observed decrease of the D-alanine pool is statistically significant for strains GPM259 ($P = 0.05$), GPM14 ($P < 0.01$), and GPM260 ($P < 0.01$). For strain mc^{2}155, the decrease of the D-alanine pool fell below the statistically significant level ($P = 0.11$). However, this strain displayed the maximum accumulation of L-alanine, and the L-alanine-to-D-alanine molar ratio increased 3.5-fold ($P = 0.01$).

The intracellular pools of D- and L-alanine were also dependent on the steady-state production levels of Alr and Ddl enzymes. In cells not exposed to DCS, a twofold excess of the L-stereoisomer was observed for strains mc^{2}155, GMP259, and GPM 260 ($P = 0.30$) but strain GPM14 displayed almost equimolar amounts of both isomers, a result significantly different from those for the rest of the strains ($P = 0.01$). These data suggest that the overproduction of Alr favors the maintenance of a relatively abundant pool of D-alanine. Treatment with DCS led to the most significant changes, as observed by comparing effects among different strains. In the wild-type strain mc^{2}155, an eightfold excess of L- over D-alanine was observed. In the Alr-overproducing strain GPM14, only a three-fold excess was observed, representing a statistically significant difference from the wild-type strain ($P = 0.02$). In contrast, the Ddl-overproducing strains GPM259 and GPM260 displayed an 18- to 20-fold excess of L-alanine. This result indicated that there were no significant differences between

GPM259 and GPM260 ($P = 0.80$) but that these two strains differed significantly from both the wild-type strain ($P = 0.04$) and GPM14 ($P = 0.01$). Furthermore, these changes are mostly determined by a significant reduction in the D-alanine pool compared to the values for the wild-type strain mc^{2}155 ($P = 0.03$) and GPM14 ($P < 0.01$). These observations suggest that, in the Ddl-overproducing strains GPM259 and GPM260, Ddl is not significantly inhibited by DCS at concentrations that inhibit Alr. Moreover, the inhibition of Alr by DCS was quite effective, since the Alr-Ddl-overproducing strain GPM260 displayed values for both the D- and L-alanine pools similar to those displayed by strain GPM259, with wild-type Alr levels.

DISCUSSION

In this study, we demonstrated that Ddl enzyme activities in crude extracts were inhibited by DCS in a concentration-dependent manner, similar to the effect we observed for the Alr enzyme (5). Approximately 50% of Ddl enzyme activity is inhibited by DCS at a concentration of 200 μg/ml, while only 10 to 15% of the Ddl activity is inhibited at 50 μg/ml, near the MIC. The apparent discrepancy reflects the difference between in vivo and in vitro conditions. In live bacilli, DCS also inhibits Alr, resulting in a limited supply of D-Ala, which is the substrate for Ddl, while in the cell-free assay, D-Ala is provided in excess. These data confirmed that the mycobacterial Ddl enzyme is a target of DCS.

Overexpression of either the *M. smegmatis* or *M. tuberculosis ddl* gene, using the same expression vectors as those used to overexpress Alr (5), confers an intermediate level of resistance to DCS. Furthermore, GPM260, a recombinant strain of *M. smegmatis* overproducing both Alr and Ddl, was constructed and characterized. GPM260 displayed a higher level of resistance to DCS than its parent strain, GPM14, which is consistent with the increased DCS resistance levels of *S. gordonii* mutants with elevated Alr and Ddl activities (22). To test the hypothesis that inhibition of Alr by DCS decreases the intracellular pool of D-alanine, we compared the levels of D-alanine pools in wild-type and recombinant *M. smegmatis* strains with or without DCS treatment. The results showed that DCS treatment decreased the intracellular D-alanine pools in strains with wild-type Alr activity while the Alr-overproducing strain GPM14 maintained a relatively abundant pool of D-alanine. This observation is consistent with the intermediate level of resistance to DCS of Ddl-overproducing strains. In addition, analysis of the alanine pools in Alr-Ddl-overproducing strain GPM260 suggests that, upon exposure to DCS, Alr is readily inhibited. In contrast, overproduction of Alr and/or Ddl does not have an effect on the susceptibility to vancomycin. The increased resistance of a previously isolated *M. smegmatis* mutant to both DCS and vancomycin (20) may underscore a resistance mechanism different from overproduction of Alr and/or Ddl. Regarding resistance to DCS, there has been only one fully characterized mechanism in mycobacteria involving overexpression of the *alr* gene (5). In this study, we have shown that overexpression of the *ddl* gene also confers DCS resistance in *M. smegmatis*, but at levels of resistance below those for strains overproducing Alr. Therefore, we do not expect that mutational changes, for example, *ddl* promoter-up mutations, would play an important role in the emergence of naturally DCS-resistant strains. The presence of a mutation(s) in the *ddl* structural gene leading to DCS resistance has not been demonstrated and needs further investigation.

DCS targets both Alr and Ddl enzymes, but the lethal target for its bactericidal effect has not been identified. *M. smegmatis alr* null mutants are not dependent on D-alanine for growth, indicating that there is another pathway for D-alanine biosynthesis (6). In addition, the *M. smegmatis alr* mutant is hypersusceptible to DCS, underlying the existence of another lethal target, most likely Ddl. The DCS hypersensitivity phenotype of the *alr* mutant is also consistent with the essentiality of the *M. smegmatis ddl* gene, as suggested by the temperature sensitivity phenotype of a mutant carrying a single amino acid substitution in Ddl (3). This temperature-sensitive mutant is also more susceptible to DCS, presumably due to the low activity of the mutated Ddl. Therefore, Ddl seems to be the lethal target of DCS, while the inhibition of Alr by DCS further decreases the D-alanine pool and contributes to the inhibition of Ddl. Previously, it was suggested that DCS resistance in *M. tuberculosis* is primarily due to mutations in the *ddl* gene (9), and it was further speculated that inhibition of Alr plays only a minor role in the mechanism of DCS action (8). Contrary to this, our data showed that Ddl is not significantly affected by DCS at the concentration that inhibits Alr. Our observation suggests that Alr overproduction contributes to the maintenance of the internal D-alanine pool, thus antagonizing the inhibition of Ddl by DCS. For the *Streptococcus faecalis* enzymes, the DCS inhibition constants for both Alr ($K_i = 0.02$ mM) and Ddl ($K_i = 0.9$ mM) have been determined from the purified enzymes (19). If the K_is for the mycobacterial enzymes were to follow a similar trend, an attractive hypothesis would be that the overproduction of a high-affinity DCS-binding target (Alr) would protect a more fundamental and low-affinity target, probably Ddl, from drug inhibition. In this view, the major mechanism of resistance is the overproduction of the high-affinity but dispensable target, while the bactericidal effect is due to the inhibition of the low-affinity lethal target. Our studies do not rule out the possibility that the bactericidal action of DCS may result from the inhibition of a lethal target(s) other than Ddl. Further research is necessary to assess the lethal targets in the D-alanine branch of peptidoglycan biosynthesis in mycobacteria. This information is necessary for the development of new antimycobacterial agents targeting the D-alanine pathway.

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REFERENCES

1. **Bashyam, M. D., and A. Tyagi.** 1994. An efficient and high-yielding method for isolation of RNA from mycobacteria. BioTechniques **17:**834–836.
2. **Belanger, A. E., and J. M. Inamine.** 2000. Genetics of cell wall biosynthesis, p. 191–202. *In* G. F. Hatfull and W. R. J. Jacobs (ed.), Molecular genetics of mycobacteria. ASM Press, Washington, D.C.

3. **Belanger, A. E., J. C. Porter, and G. F. Hatfull.** 2000. Genetic analysis of peptidoglycan biosynthesis in mycobacteria: characterization of a *ddlA* mutant of *Mycobacterium smegmatis*. J. Bacteriol. **182:**6854–6856.
4. **Brennan, P. J., and H. Nikaido.** 1995. The envelope of mycobacteria. Annu. Rev. Biochem. **64:**29–63.
5. **Caceres, N. E., N. B. Harris, J. F. Wellehan, Z. Feng, V. Kapur, and R. G. Barletta.** 1997. Overexpression of the D-alanine racemase gene confers resistance to D-cycloserine in *Mycobacterium smegmatis*. J. Bacteriol. **179:**5046–5055.
6. **Chacon, O., Z. Feng, N. B. Harris, N. E. Caceres, L. G. Adams, and R. G. Barletta.** 2002. *Mycobacterium smegmatis* D-alanine racemase mutants are not dependent on D-alanine for growth. Antimicrob. Agents Chemother. **46:**47–54.
7. **Connell, N. D., E. Medina-Acosta, W. R. McMaster, B. R. Bloom, and D. G. Russell.** 1993. Effective immunization against cutaneous leishmaniasis with recombinant bacille Calmette-Guerin expressing the *Leishmania* surface proteinase gp63. Proc. Natl. Acad. Sci. USA **90:**11473–11477.
8. **David, H. L.** 1971. Resistance to D-cycloserine in the tubercle bacilli: mutation rate and transport of alanine in parental cells and drug-resistant mutants. Appl. Microbiol. **21:**888–892.
9. **David, H. L., K. Takayama, and D. S. Goldman.** 1969. Susceptibility of mycobacterial D-alanyl-D-alanine synthetase to D-cycloserine. Am. Rev. Respir. Dis. **100:**579–581.
10. **David, S.** 2001. Synergic activity of D-cycloserine and ss-chloro-D-alanine against *Mycobacterium tuberculosis*. J. Antimicrob. Chemother. **47:**203–206.
11. **Davis, L. G., W. M. Kuehl, and J. F. Battey.** 1994. Basic methods in molecular biology, 2nd ed. Appleton & Lange, Norwalk, Conn.
12. **Dutka-Malen, S., C. Molinas, M. Arthur, and P. Courvalin.** 1992. Sequence of the *vanC* gene of *Enterococcus gallinarum* BM4174 encoding a D-alanine: D-alanine ligase-related protein necessary for vancomycin resistance. Gene **112:**53–58.
13. **Farmer, P.** 2001. DOTS and DOTS-plus: not the only answer. Ann. N. Y. Acad. Sci. **953:**165–184.
14. **Foley-Thomas, E. M., D. L. Whipple, L. E. Bermudez, and R. G. Barletta.** 1995. Phage infection, transfection and transformation of *Mycobacterium avium* complex and *Mycobacterium paratuberculosis*. Microbiology **141:**1173–1181.
15. **Kaufman, D. S., and W. F. Manley.** 1998. A new procedure for determining enantiomeric (D/L) amino acid ratios in fossils using reverse phase liquid chromatography. Quat. Sci. Rev. **17:**987–1000.
16. **Lambert, M. P., and F. C. Neuhaus.** 1972. Mechanism of D-cycloserine action: alanine racemase from *Escherichia coli* W. J. Bacteriol. **110:**978–987.
17. **Manning, J. M., N. E. Merrifield, W. M. Jones, and E. C. Gotschlich.** 1974. Inhibition of bacterial growth by beta-chloro-D-alanine. Proc. Natl. Acad. Sci. USA **71:**417–421.
18. **Marshall, C. G., and G. D. Wright.** 1998. DdlN from vancomycin-producing *Amycolatopsis orientalis* C329.2 is a VanA homologue with D-alanyl-D-lactate ligase activity. J. Bacteriol. **180:**5792–5795.
19. **Neuhaus, F. C.** 1967. D-cycloserine and O-carbamyl-D-serine, p. 40–83. *In* D. Gottlieb and P. L. Shaw (ed.), Antibiotics, mechanisms of action, vol. 1. Springer-Verlag, Heidelberg, Germany.
20. **Peteroy, M., A. Severin, F. Zhao, D. Rosner, U. Lopatin, H. Scherman, A. Belanger, B. Harvey, G. F. Hatfull, P. J. Brennan, and N. D. Connell.** 2000. Characterization of a *Mycobacterium smegmatis* mutant that is simultaneously resistant to D-cycloserine and vancomycin. Antimicrob. Agents Chemother. **44:**1701–1704.
21. **Rastogi, N., K. S. Goh, and H. L. David.** 1990. Enhancement of drug susceptibility of *Mycobacterium avium* by inhibitors of cell envelope synthesis. Antimicrob. Agents Chemother. **34:**759–764.
22. **Reitz, R. H., H. D. Slade, and F. C. Neuhaus.** 1967. The biochemical mechanisms of resistance by streptococci to the antibiotics D-cycloserine and O-carbamyl-D-serine. Biochemistry **6:**2561–2570.
23. **Reyrat, J. M., and D. Kahn.** 2001. *Mycobacterium smegmatis*: an absurd model for tuberculosis? Trends Microbiol. **9:**472–474.
24. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. **Snapper, S. B., R. E. Melton, S. Mustafa, T. Kieser, and W. R. Jacobs, Jr.** 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol. Microbiol. **4:**1911–1919.
26. **Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, W. R. J. Jacobs, and B. R. Bloom.** 1991. New use of BCG for recombinant vaccines. Nature **351:**456–460.
27. **Strohl, W. R.** 1992. Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. Nucleic Acids Res. **20:**961–974.
28. **Strych, U., R. L. Penland, M. Jimenez, K. L. Krause, and M. J. Benedik.** 2001. Characterization of the alanine racemases from two *Mycobacteria*. FEMS Microbiol. Lett. **196:**93–98.
29. **Takiff, H. E., M. Cimino, M. C. Musso, T. Weisbrod, R. Martinez, M. B. Delgado, L. Salazar, B. R. Bloom, and W. R. Jacobs.** 1996. Efflux pump of the proton antiporter family confers low-level fluoroquinolone resistance in *Mycobacterium smegmatis*. Proc. Natl. Acad. Sci. USA **93:**362–366.
30. **Timm, J., M. G. Perilli, C. Duez, J. Trias, G. Orefici, L. Fattorini, G. Amicosante, A. Oratore, B. Joris, J. M. Frere, A. P. Pugsley, and B. Gicquel.** 1994. Transcription and expression analysis, using *lacZ* and *phoA* gene fusions, of *Mycobacterium fortuitum* beta-lactamase genes cloned from a natural isolate and a high-level beta-lactamase producer. Mol. Microbiol. **12:**491–504.
31. **Tyagi, J. S., and D. Sharma.** 2002. *Mycobacterium smegmatis* and tuberculosis Trends Microbiol. **10:**68–69.
32. **Walsh, C. T.** 1989. Enzymes in the D-alanine branch of bacterial cell wall peptidoglycan assembly. J. Biol. Chem. **264:**2393–2396.
33. **Whipple, D. L., R. B. Le Febvre, R. E. Andrews, Jr., and A. B. Thiermann.** 1987. Isolation and analysis of restriction endonuclease digestive patterns of chromosomal DNA from *Mycobacterium paratuberculosis* and other *Mycobacterium* species. J. Clin. Microbiol. **25:**1511–1515.
34. **Wijsman, H. J.** 1972. The characterization of an alanine racemase mutant of *Escherichia coli*. Genet. Res. **20:**269–277.
35. **World Health Organization.** 2000. Guidelines for establishing DOTS-Plus pilot projects for the management of multidrug-resistant tuberculosis (MDR-TB). WHO/DCS/TB/2000.279. World Health Organization, Geneva, Switzerland.
36. **Yew, W. W., C. F. Wong, P. C. Wong, J. Lee, and C. H. Chau.** 1993. Adverse neurological reactions in patients with multidrug-resistant pulmonary tuberculosis after coadministration of cycloserine and ofloxacin. Clin. Infect. Dis. **17:**288–289.
37. **Zygmunt, W. A.** 1963. Antagonism of D-cycloserine inhibition of mycobacterial growth by D-alanine. J. Bacteriol. **85:**1217–1220.